NAVAL HEALTH RESEARCH CENTER

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Report No. 99-6

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The Effect of Vitamin Supplementation on Oxidative Stress During Strenuous Cold Weather Training at Moderate Altitude

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Report No. 99-6, supported by the Office of Naval Research, Department of the Navy under Research Work Unit 63706N.M0096.002-6603. The views expressed in this article are those of the authors and do not reflect official policy or position of the Department of the Navy, Department of Defense, or the U.S. Government. Approved for public release, distribution unlimited.

Human subjects participated in this study after giving their free and informed consent. Investigators adhered to NAVHLTHRSCHCENINST 3900.2, 2 Aug 95, concerning the protection of human volunteers in medical research.

The assistance of U.S. Marine Corps personnel as subject volunteers for this study is acknowledged and appreciated. The authors wish to thank Ross Laboratories for a grant to support this research.

Executive Summary

Problem: Oxidative stress is a term used to denote an excessive amount of reactive oxygen species in the body produced by aerobic exercise, hypoxia, ultraviolet (UV) light, and pollution. The long-term production of reactive oxygen species can have deleterious effects termed oxidative injury. Since most military field operations involve one or more of these factors (e.g., physical work, altitude, and UV exposure) it is probable that military personnel have an elevated risk of oxidative stress that could lead to oxidative injury. Oxidative injury involves cell membrane alterations, decreased membrane fluidity, inability to maintain ionic gradients, cellular swelling, tissue inflammation, deoxyribonucleic acid (DNA) damage, and protein changes. The immune system is very susceptible to oxidative stress leading to immune function depression.

Objective: The purpose of this study was to quantify the antioxidant capability of α-tocopherol, ascorbic acid, and β-carotene, used either singly or in combination, to reduce oxidative stress in U.S. Marine Corps personnel undergoing winter training at moderate altitude. The first objective was to quantify the oxidative stress by indirect measures of lipid peroxidation and DNA alteration. The second objective was to assess the efficacy of the vitamins to modify the urinary, blood, and breath indices of lipid peroxidation.

Approach: Seventy-five Marines involved in Winter Warfare Training at the Marine Corps Mountain Warfare Training Center (MCMWTC), Bridgeport, CA, were recruited and placed in 1 of 5 groups for a double-blind 14-day supplementation study. The groups were: (1) group 1 receiving 500 mg ascorbic acid daily; (2) group 2 receiving 400 IU α-tocopherol daily; (3) group 3 receiving 20,000 IU β-carotene daily; (4) group 4 receiving 500 mg ascorbic acid, 400 IU α-tocopherol, 20,000 IU β-carotene, 100 μg selenium, and 30 mg zinc daily; and (5) placebo group receiving 1,000 mg oyster shell calcium daily. Oxidative stress indicators included plasma lipid peroxides and oxygen radical absorption capacity, urine malondialdehyde and 8-hydroxy-deoxy-guanosine, and breath pentane. Samples were collected before the Marines were transported to MCMWTC, Day 0 at 6,700 ft, Day 6 and 12 at 9,200 ft, and at Day 14 at 6,700 ft. Energy intake, coupled with body composition and weight changes, provided an estimate of energy expenditure.

Results: The mean daily energy expenditure during the 14 days of field training was 5,500 kcal with an average weight loss of 7.5 lb and 1.6 percent body fat. Both the placebo and single antioxidant groups showed increased oxidative stress, indicated by elevated breath pentane. The antioxidant mixture group showed a much lower increase in oxidative stress during the same period. Other measures of oxidative stress indicated oxidative stress was increased but did not reflect the same differentiation of supplementation regimens as did the breath pentane.

Conclusions: Breath pentane, plasma lipid peroxides, urine malondialdehyde, and 8-hydroxy-deoxy-guanosine generally reflected increased oxidative stress. The individual antioxidants were relatively ineffective in reducing the indicators of oxidative stress. The mixture of antioxidants produced significantly lower oxidative stress, as shown by breath pentane, during the 14-day training. Individual variation of response is the leading reason for nonsignificant results in the

other measures of oxidative stress. People who work and recreate at moderate altitude may be at increased risk for oxidative injury due to free radical production.

Recommendations: This model provides a good representation of the oxidative stress load encountered during military field operations and, therefore, it is an ideal platform for further testing of antioxidant supplementation to minimize oxidative stress. Larger groups and longer supplementation times are needed to reduce the variability of response. Refinement in the timing and manner of sample collection also would maximize the information gained from new studies.

Introduction

Oxidative stress is a term used to denote an excessive amount of reactive oxygen species (ROS) in the body produced by aerobic exercise, hypoxia, ultraviolet (UV) light, and pollution. The long-term production of ROS can have deleterious effects termed oxidative injury. ROS can damage most cell components through initiation of a lipid peroxidation chain reaction (Alessio, 1993; Haramaki & Packer, 1994). Oxidative stress leads to cell membrane alterations, decreased membrane fluidity, inability to maintain ionic gradients, cellular swelling, tissue inflammation, deoxyribonucleic acid (DNA) damage, and protein changes (Alessio, 1993; Haramaki & Packer, 1994). The best known chronic effect of oxidative injury is premature aging associated with high levels of UV exposure. The symptoms of exercise-induced oxidative injury include muscle soreness, delayed onset muscle soreness, fatigue, and increased injury recovery time. Ingesting vitamins that act as antioxidants may augment the body's cellular antioxidant defense system and decrease the short-term tissue damage and long-term health risk. At higher altitudes, the oxidative stress may be greater due to a combination of causative factors: hypoxia, increased metabolic rate, increased intensity of UV light, cold temperatures, and large temperature changes.

Mechanisms of ROS production

Free radicals are molecules or fragments of molecules containing an unpaired electron (e) in the valence shell. Free radicals are a natural part of biological systems and most are associated with the presence of molecular oxygen (O_2). The mitrochondrion is the subcellular site for the final stages of biological oxidation. Normally, respiratory oxidation occurs by removing hydrogen ions (H^+), transferring electrons along a series of coupled electron transport redox components, and ultimately adding O_2 to form water. During increased O_2 use (e.g., during exercise), there is evidence that ROS production increases (Alessio, 1993; Kanter, 1994; Clarkson, 1995). When O_2 metabolism is increased, 4% to 5% of the electrons removed during the metabolism of substrates can escape from the electron transport chain and reduce O_2 directly giving rise to superoxides.

Exercise: The probable mechanism of ROS production during exercise is "electron leakage." During exercise, localized O_2 uptake in skeletal muscle can increase 10 to 40 times above resting levels (Askew, 1995; Singh, 1992). This increased O_2 uptake can exceed the capacity of mitrochrondrial oxidative activity in the cytochrome system to properly reduce O_2. As a result, the electron transport chain leaks a few electrons, which are picked up by O_2, forming the superoxide radical while passing the bulk of the electrons onto the next component of the cytochrome system.

Reduction of O_2 requires four single electron-transfer reactions and four H^+ to convert O_2 to water. When fewer than four electrons are available, the following ROS can be formed:

eq1: $O_2 + 1e \rightarrow {}^*O_2^-$ (superoxide radical)
eq2: $O_2 + 2e + 2H^+ \rightarrow H_2O_2$ (hydrogen peroxide)
eq3: $O_2 + 3e + 3H^+ \rightarrow H_2O + {}^*OH$ (hydroxyl radical)
eq4: $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^*OH + OH^-$

"*" denotes the existence of an unpaired electron in the outer orbit of the radicals. The superoxide ion and hydrogen peroxide are the main products of the electron leakage. The main site of leakage occurs at the beginning of the nicotinamide adenine dinucleotide phosphate-coenzyme quinone-reductase complex in the cytochrome system at the mitrochrondrial level (Halliwell & Gutteridge, 1993).

The superoxide ion is unstable and protonates in the dismutation reaction to form the hydroperoxyl radical:

eq5: $*O_2^- + H^+ \rightarrow$ HOO* (hydroperoxyl radical)

The hydroperoxyl radical is very reactive and initiates a chain reaction by removing a hydrogen atom from methylene groups (Halliwell & Gutteridge, 1993).

eq6: $R\text{-}CH_2\text{-}R + HOO^* \rightarrow R\text{-}C^*H\text{-}R$ (carbonyl radical)$+ H_2O_2$

The methylene group that is attacked can be part of proteins, lipids, phospholipids, or other long chain carbon species. The most accessible targets for the hydroperoxyl radical are the methylene groups of polyunsaturated fatty acids in biological membranes (Halliwell & Gutteridge, 1993; Mayes, 1990). The removal of a H^+ from a methylene group (proteins, phospholipids, or other carbon species) forms H_2O_2 and leaves an unpaired electron on carbon (carbonyl radical). The carbonyl radical stabilizes by molecular rearrangement to form a conjugated diene:

eq7: $R\text{-}C^*H\text{-}R$ (carbonyl radical) $\rightarrow H_3C\text{–}C^*H\text{-}R$ (diene)

eq8: $H_3C\text{-}C^*H\text{-}R + O_2 \rightarrow H_3C\text{-}CH(O^*)\text{-}R$ (peroxyl radical)

The conjugated diene combines with O_2 to form the peroxyl radical, which reacts with a polyunsaturated fatty acid to form a lipid hydroperoxide and a new carbonyl radical (Halliwell & Gutteridge, 1993). In the presence of ferrous iron, lipid hydroperoxides will form alkoxyl radicals, which subsequently form the hydrocarbons ethane and pentane.

In the presence of O_2, hydroperoxides form cyclic peroxides and cyclic endoperoxides. Through iron-induced (presence of Fe^{2+}) fragmentation of cyclic endoperoxides, malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and other aldehydes and alkenals are formed (Halliwell & Gutteridge, 1993).

The H_2O_2 formed during the production of the carbonyl radical (eq6) is important because it readily decomposes to the hydroxyl radical in the presence of ferrous iron and copper.

The several ROS that can be formed during increased O_2 use are: peroxyl radical, superoxide radical, hydroxyl radical, alkoxyl radical, phenoxyl, and semiquinone radicals. The various ROS are able to attach to and initiate free radical chain reactions in membrane lipids, proteins, and DNA throughout the body. The hydroxyl radical attacks the DNA bases thymine and guanine to produce mutagenic nucleosides. Extensive DNA damage can cause mutations, strand breaks, and cell death (Halliwell & Gutteridge, 1993).

Hypoxia: Local tissue hypoxia is a common occurrence in muscles during exercise (Witt, Reznick, Viguie, Starke-Reed, & Packer, 1992). Hypoxic cells become particularly susceptible to oxidative stress since mitochrondria lack enough O_2 to accept all available electrons. Some electrons escape from the cytochrome system and are transported to O_2 upon tissue reoxygenation after exercise intensity decreases. This produces a burst of superoxide and hydrogen peroxide production.

Ultraviolet Light (UV): UV light energy consists of three wavelength bands. The band wavelengths are: UVC (100 to 280 nm), UVB (280 to 315 nm), and UVA (315 to 400 nm). Since most of the UV energy in the range of 100 to 290 nm is absorbed and scattered by the earth's atmosphere, the earth surface and its inhabitants are exposed to UV light in the range 290 to 400 nm (World Health Organization, 1993). The UV light not scattered or reflected by the skin penetrates the dermis and even reaches the subcutaneous layer (World Health Organization, 1993). About 1% of the longer wavelengths of UVA light penetrates to the subcutaneous layer. This irradiation induces lipid peroxidation and suppression of DNA synthesis due to DNA damage related to the UV-induced free radical generation within the cells (World Health Organization, 1993). UV light exposure requires antioxidant defenses to neutralize the free radicals that arise in skin cells and red blood cells.

UV light causes cellular damage by the development of singlet molecular oxygen. Singlet molecular oxygen, which is not classified as a free radical, is produced by UV light causing ionization of the outer electron of O_2. However, the presence of an excited unpaired electron in its outer orbit enables singlet molecular oxygen to attack unsaturated fatty acids removing a H^+ Askew, 1995). Singlet molecular oxygen can be a significant source of oxidative stress in skin exposed to intense sunlight.

Altitude: As altitude increases, barometric pressure decreases, causing a decrease in the partial pressure of O_2 (pO_2) in inhaled air. The partial pressure of O_2 at sea level is 149 mmHg and is 101 mmHg at 9,200 ft (altitude of training area at Marine Corps Mountain Warfare Training Center [MCMWTC]). Oxygen enters the lungs where it binds with hemoglobin for transport to the tissues. A reduced pO_2 reduces the oxygenation of blood flowing through the lungs. This decreased pO_2 results in the delivery of less oxygenated blood to the tissues, thus creating tissue hypoxia. Another source of increased oxidative stress at altitude is the increased intensity of UV sunlight. UVB is more susceptible to atmospheric scattering than UVA because of its shorter wavelength; thus incident UVB is attenuated less in clean air at higher altitudes. For every 1,000-m increase in altitude, UVB intensity increases by 15% to 20%, while UVA intensity increases 7% to 10% (Simon-Schnass, 1994). This results in an increase of 350% to 520% in incident UVB energy and a 185% to 240% increase in incident UVA energy at MCMWTC. Increased UV exposure can lead to the production of the ROS singlet molecular oxygen.

Indices of Lipid Peroxidation

In humans, there is no direct measure of lipid peroxidation. However, several indirect methodologies do exist. The current trend in research is to employ two or more indirect measurements of lipid peroxidation to increase validity. The indices used in this study focused on the following: plasma lipid hydroperoxides (LPO), serum oxygen radical absorption capacity (ORAC), urine total aldehydes (MDA and 4-hydroxynonenal [4-HNE]), urine 8-hydroxy-deoxy-guanosine (8-OHdG), urine 8-epi-prostaglandin factor (8-epi-$PGF_{2\alpha}$), and breath pentane.

Lipid hydroperoxides: LPO is the hydrolyzed lipid peroxide product of triacylgycerides and phospholipids. LPO is measured in serum or plasma using lipoprotein lipase to hydrolyze oxidized triglycerides and phospholipids to lipid hydroperoxides. This method uses high-pressure liquid chromatography (HPLC) to measure hydroperoxides, their precursors (peroxyl radicals, carbonyl radicals, and cyclic endoperoxides), and their unbound fragments (MDA, 4-hydroxyalkenals, and other aldehydes).

Serum oxygen radical absorption capacity (ORAC): The ORAC assay is a method that utilizes the free radical sensitive fluorescent indicator protein β-phycoerythrin (β-PE) to measure the efficacy of endogenous serum antioxidants to protect β-PE from damage by free radicals (Cao, Allessio, & Cutler, 1993). On a molar basis, β-PE protein reacts with ROS over 100 times slower than most biological antioxidants, such as thiols, uric acid, bilirubin, and ascorbate. Therefore, all active antioxidants are completely oxidized before the β-PE protein starts to become oxidized (Delang & Glazer, 1989). Serum ORAC is a method for measuring total serum antioxidant activity.

Urine total aldehydes: MDA and 4-hydroxyalkenals are metabolic end products from peroxidation of polyunsaturated fatty acids (PUFA) and related esters (Esterbauer, Schaur, & Zollner, 1991). Total aldehydes can be measured in both urine and serum. The hydroxyalkenal 4-HNE, is the major product of the peroxidation decomposition of ω-6 PUFA and possesses cytotoxic, hepatotoxic, mutagenic, and genotoxic properties (Esterbauer & Cheeseman, 1990). Increased concentration of 4-HNE have been found in plasma and organs under conditions of oxidative stress. MDA is the most abundant aldehyde resulting from lipid peroxidation.

Urine 8-hydroxy-deoxy-guanosine: 8-OHdG is an end product (C-8 hydroxylation of guanine) of DNA damage by ROS. Damaged nucleic acid bases are removed from the DNA strand by the repair enzyme, DNA polymerase I, and are excreted in the urine. This DNA modification has earned much interest due to its mutagenic potential and has been commonly used as a biological marker for oxidative DNA damage. 8-OHdG assay involves use of an immunosorbent assay for the oxidative DNA adduct 8-OHdG. Quantification of 8-OHdG is achieved by comparison with optical densities of the chromatogenic signal of the sample with a standard curve of 8-OHdG concentrations.

Urine 8-epi-prostaglandin factor: 8-epi-$PGF_{2\alpha}$ is a specific nonenzymatic lipid peroxidation product of a prostaglandin derived from arachidonic acid. It has been suggested that elevated circulating concentrations of 8-epi-$PGF_{2\alpha}$ may contribute to the pathology of hepatorenal

syndrome caused by oxidative injury (Roberts & Morrow, 1996). It has also been reported that noninsulin dependent diabetes mellitus is associated with an increased plasma concentration of esterified 8-epi-$PGF_{2\alpha}$ (Gopaul, Anggard, Mallet, Betteridge, Wolff, & Nourooz-Zadeh, 1995). These studies indicate that the use of 8-epi-$PGF_{2\alpha}$ as a marker of oxidative stress in vivo may be a major advance in the exploration of the role of ROS in the pathogenesis of human disease.

Breath Pentane: Pentane is formed from the pentanyl radical that is the breakdown product of ω-6 PUFA (linoleic or arachidonic acid) (Pincemail, Deby, & Dethier, 1987). Pentane is transported via the blood to the lungs where it is expired in the breath. Pentane production during exercise has been reported to increase linearly with exercise intensity (Haramaki & Packer, 1994). A major limitation of measuring breath pentane in humans is that isoprene, a normal component of human breath, and pentane co-elute on most gas chromatograph columns. Successful use of breath pentane as a marker of oxidative stress requires the separation of these peaks.

Antioxidant Properties

All aerobic cells maintain oxidative stress defense systems composed of cellular and extracellular antioxidants. ROS are blocked at various stages of formation and during reactions with biomolecules. The three main components of antioxidant defenses are: antioxidant enzymes (superoxide dismutase, catalase, and peroxidase) and mineral cofactors (selenium, magnesium, zinc, and manganese), antioxidant compounds (glutathione, uric acid, and taurine), and antioxidant vitamins (alpha tocopherol, ascorbic acid, and β-carotene).

Alpha tocopherol (vitamin E): Vitamin E is a fat-soluble vitamin found in membranes throughout the body. The recommended daily allowance (RDA) for vitamin E is 10 mg for men and 8 mg for women. Vitamin E antioxidant properties include the ability to preserve membrane integrity by breaking the chain reaction of peroxidation of fatty acids in the membranes and preventing oxidation of cholesterol and proteins. Vitamin E is a scavenger of free radicals and is the first line of defense against peroxyl radicals (Kanter, 1994; Simon-Schnass, 1994; Mayes, 1990). It has been reported that a daily supplementation of 300 mg of vitamin E reduced serum MDA in cyclists (Sumida, Tanaka, Kitao, & Nakadomo, 1989). During a 6-week transpolar ski trek, it was reported that as plasma and erythrocyte tocopherol decreased, plasma MDA increased (Panin, Mayaskaya, & Borodin, 1992).

Ascorbic acid (vitamin C): Vitamin C is a water-soluble antioxidant found mainly in plasma and in the aqueous medium of the cell. The RDA for vitamin C is 60 mg for adults. It has a strong reducing potential that is capable of scavenging a wide variety of ROS. Vitamin C scavenges superoxide radicals, hydroxyl radicals, peroxyl radicals, H_2O_2, and singlet molecular oxygen. Vitamin C deactivates the extracellular oxidants generated by activated neutrophils and also suppresses the activity of (quenches) free radicals that diffuse out of the extracellular space (Sen & Hanninen, 1994). Kaminski and Boal (1992) found that 3,000 mg of vitamin C given before and after an exercise bout reduced the development of muscle soreness. Vitamin C supplementation has been reported to cause significant reductions of lipid peroxidation (Frei, England, & Ames, 1989; Link, Pinson, Kahane, & Hersko, 1987).

β-carotene: β-carotene is a fat-soluble pro-vitamin form of vitamin A found in vegetables as a yellow pigment. The RDA for β-carotene is 167 μg for men and 134 μg for women. β-carotene has been found to be an effective chain-breaking antioxidant and an efficient quencher of singlet molecular oxygen. It has been reported that β-carotene was effective in reducing lipid peroxidation as indicated by reductions in MDA (Gottlieb, Zarling, Mobarhan, Bowen, & Sugerman, 1993).

In summary, it is clear that increased oxygen uptake, hypoxia, and increased UV radiation can increase the incidence of oxidative stress and may lead to cellular oxidative injury. Since most military field operations involve one or more of these factors (e.g., physical work, altitude, and UV exposure) it is probable that military personnel have an elevated risk of oxidative stress that could lead to oxidative injury. The increased physical demands of operating in hypoxic environments are sufficient to increase the risk of injury. The only method of increasing antioxidant defenses is to augment them by supplementation with vitamins having antioxidant capabilities. Since there is disagreement on the efficacy of single vitamins or vitamins used as mixtures, this study compared the response of both single vitamins and mixtures of vitamins under the same conditions of elevated oxidative stress. It has been hypothesized that some antioxidant vitamins can act as pro-oxidants, which produce more hydroxyl radicals and drive the chain reaction to create more hydroperoxides. This process utilizes enough ferrous iron so there is an insufficient amount of ferrous iron to drive hydroperoxide reactions leading to MDA and HNE. Therefore, it is important to rule out any adverse reactions of individual antioxidants before including these compounds in an antioxidant mixture.

Purpose of Study:

The purpose of this study was to evaluate the ability of α-tocopherol, ascorbic acid, and β-carotene, ingested either singly or in combination, to reduce oxidative stress in U.S. Marine Corps personnel undergoing cold weather training at moderate altitude. The first objective was to evaluate oxidative stress by indirect measures of lipid peroxidation and DNA alteration. The second objective was to assess the efficacy of the vitamins to reduce urine, plasma, and breath indices of oxidative stress.

Methods

Subjects: Seventy-five male Marines (mean age 24.2 ± 6.2 years) from the second battalion of the fifth Marines from Camp Pendleton, CA, who enrolled in the March 97 class of MCMWTC participated in a randomized double-blind study during cold weather field training. Test subjects were briefed on the purpose of the study and informed consent was secured from each of the 75 volunteers according to the provisions of NAVHLTRSCHCENINST 3900.2. Subjects were informed that they could withdraw from the study at any time without loss of benefits or penalty. Each volunteer was then randomly assigned to 1 of 5 groups. The five study groups were:

1) Treatment group 1, which self-administered two tablets daily providing a total of 500-mg ascorbic acid during the study.

2) Treatment group 2, which self-administered two tablets daily providing a total of 400 IU α-tocopherol (1.5 IU α-tocopherol = 1.0 mg) during the study.
3) Treatment group 3, which self-administered two tablets daily providing a total of 20,000 IU β-carotene (1 IU β-carotene = 0.6 μg) during the study.
4) Treatment group 4, which self-administered two tablets daily providing mixture of 500 mg ascorbic acid, 400 IU α-tocopherol, 20,000 IU β-carotene, 100 μg selenium, and 30 mg zinc during the study. The dosage of each treatment group was similar to individual antioxidant content of a mixture utilized in a previous study (Pfieffer, Askew, Roberts, Wood, & Freedman, 1997) to provide an opportunity for comparison of results between the studies.
5) Placebo control group, which self-administered two tablets daily providing 1,000-mg oyster shell calcium during the study.

Data Collection: There were five data collection periods:

1) Camp Pendleton, March 20, 1997 - sea level. Breath, blood (for serum carotenoids, vitamin E, and vitamin C profiles), and urine samples were collected. Vitamin supplementation started after this collection period and continued until the end of field training.
2) Day 0, April 8, 1997, - 6,700 ft at base camp, MCMWTC. Breath, blood, and urine samples were collected. Body weight and skinfold data were obtained.
3) Day 6, April 14, 1997, - 9,200 ft at the Silver Creek Training Site (SCTS), MCMWTC. Breath and urine samples were collected. Three-day dietary intake records were obtained on days 8, 9, and 10.
4) Day 12, April 20, 1997, - 9,200 ft SCTS, MCMWTC. Breath and urine samples were collected.
5) Day 14, April 22, 1997, - 6,700 ft at base camp immediately following return from SCTS, MCMWTC. Breath, blood, and urine samples were collected. Body weight and skinfold data were obtained.

Body weight was measured by electronic scale (AND, model HV-150K; San Diego, CA). Each subject wore briefs and no socks for the weighing. Body composition was determined from skinfold and body circumference measurements using established equations (Durnin & Wormsley, 1974). Skinfold calipers (Harpenden Skinfold Caliper; Holland, MI) were used to measure skinfold thickness at three sites on each subject (chest, abdomen, and thigh) in two consecutive sets, and then the measurements at each site were averaged.

Diet: Meals, during the field training, were: (1) Meals, Ready to Eat (MRE), which are the principle packaged military field ration; (2) Ration, Cold Weather (RCW), which are military field rations that are specially developed to withstand low temperature; or (3) hot meals prepared in the base-camp mess hall and delivered to the field in insulated mermite containers via tracked vehicle. Four MREs for 1 day provided 5,200 kcal/day (21,800 kJ/day), whereas one RCW provided 4,500 kcal (18,800 kJ) for 1 day. Hot meals from base camp were delivered several times for the evening meal during the first week of field training, but not utilized during the second week of training.

Three-day dietary intake records obtained during the first week at SCTS were used to estimate energy intake. Appropriate food record cards were issued for either MREs or RCWs. If

hot mess hall-prepared rations (hot-wets) were served, visual estimation of portion sizes was used in conjunction with the menu to estimate energy intake. The food analysis software, Nutritionist IV, was used to analyze the 3-day food records. Detailed information of nutrient breakdown for MREs and RCWs was supplied by the U.S. Army Research Institute for Environment Medicine, Natick, MA. These data were added into the Nutritionist IV software. Energy intake coupled with change in body weight was used to estimate energy expenditure of the 2-week field training. For the intake-balance method, weight change was calculated by the subtraction of pretraining body weight from posttraining weight. It was estimated that every pound of weight loss was approximately equivalent to 3,500 kcal. Therefore, every 1 pound of weight loss within the 2-week training was equal to an approximate daily 250-kcal energy expenditure over energy intake. This estimate was added to daily energy intake to yield an estimate of daily energy expenditure.

Laboratory Analyses

Breath pentane: Breath samples were obtained from each volunteer using a commercial device that traps and discards the initial 700 ml of expired air and then collects the subsequent deep alveolar exhalation in a tedlar bag (GaSampler, Quintron; Milwaukee, WI). Two hundred fifty ml of the air sample was then trapped using an Entech 7,000 three-phase microscale purge and trap concentrator. Organic material in the breath sample (including pentane) then was concentrated on a glass bead trap that was cryogenically cooled to -150°C. Breath pentane was measured by Data Chem Laboratories (Salt Lake City, UT) by gas chromatography (Hewlett-Packard 5890 GC with flame ionization detection [Column: Chrompack PLOT-U 25m X 0.32 mm ID; Dector: FID at 220°C; Air: 250 KPa; Hydrogen: 110 Kpa]).

Blood: Blood samples (10 ml each time for a total of 30 ml) were collected into vacutainer tubes containing EDTA by Navy Medical Corpsmen using aseptic techniques. Plasma was separated following centrifugation at 2,000 to 3,000- rpm (1,000-1,500 G) for 10 min. Plasma was transferred to cryogenic tubes (2 ml), wrapped in aluminum foil, and frozen over dry ice. All plasma samples were packed in dry ice and shipped to Ross Products Division, Abbott Laboratories (Columbus, OH) for plasma total lipid hydroperoxides analysis.

Plasma total lipid hydroperoxides were measured using a commercial kit from Kamiya Biochemical Company (Thousand Oaks, CA). In plasma total lipid hydroperoxides analysis, oxidized triglycerides and phospholipids in plasma were hydrolyzed to liberate fatty acid hydroperoxides using lipoprotein lipase. The fatty acid hydroperoxides then were reacted with a reducing agent, 10-N-ethylcarbamoyl-3, 7-dimethylamino-10H-phenothiazine (MCDP), forming the colored product, methylene blue, which was measured spectrophotometrically at 675 nm. Quantitation was achieved using a calibration curve of pure standard, 15 (S)-hydroperoxy-eicosapentaenoic acid. Plasma total LPO was expressed as micromoles of lipid peroxide per liter of plasma (Ohishi, Ohkawa, Miike, Tatano, & Yagi, 1985).

Serum samples were obtained with a clot-activated (10 ml tiger top; total = 30 ml) vacutainer tube. After clotting in subdued light, the sample was centrifuged at 2,000 to 3,000 rpm (1,000-1,500 G) for 10 min. Serum was transferred into cryogenic tubes (2 ml) and frozen

over dry ice. Samples were shipped to Genox laboratories (Baltimore, MD) for analyses of the following parameters:

1) α-tocopherol, ascorbic acid, and β-carotene concentrations were measured by HPLC on a Hewlett Packard 1090 HPLC system monitoring α–tocopherol at 292 nm and retinol at 320 nm (Stacewicz-Sapuntakis, Bowen, Kikendall, & Burgess, 1987). Serum ascorbic acid was analyzed by a colormetric procedure using ferrozine, disodium salt (McGown, Rusnak, Lewis, & Tillotson, 1982). The assay is based on the reduction of Fe^{+3} to Fe^{+2} by ascorbic acid, followed by chromogenic chelation of Fe^{+2} with ferrozine. Absorbance was read at 562 nm after approximately 15 s in the spectrophotometer.
2) Serum iron, transferrin, and the serum total iron-binding capacity (TIBC): Transferrin-bound serum iron dissociates to form ferrous ions at acid pH (4.5) and in the presence of suitable reducing agent, hydroxylamine hydrochloride. Hydroxylamine hydrochloride reacted with ferrozine to produce a magenta colored complex with an absorption maximum near 560 nm. The serum iron concentration was determined by the difference in color intensity at this wavelength, before and after addition of ferrozine. At alkaline pH (8.1), ferrous ions added to serum bind specifically with transferrin at unsaturated iron-binding sites. Remaining unbound ferrous ions were measured with the ferrozine reaction. The difference between the amount of unbound iron and the total amount added to serum was equivalent to the quantity bound to transferrin. This was the unsaturated iron-binding capacity (UIBC). TIBC equaled the total iron plus UIBC (Persijn, Van Der Slik, & Rlethorst, 1971).
3) Total aldehydes: Lipid Peroxidation Assay Kit (Calbiochem; San Diego, CA), containing Reagent 1 (R1) - N-methyl-2-phenylindole (10.3 mM) and Reagent 2 (R2) - methanesulfonic acid (15.4 M), reacted with serum MDA+4-HNE at 45°C. Condensation of one molecule of MDA or 4-HNE with two molecules of R1 yields a stable chromophore with maximal absorbance at 586 nm (Esterbauer & Cheeseman, 1990).
4) Serum total ORAC: This measure of the total antioxidant capacity was determined by fluorescence of the oxidized β–PE indicator protein (Cao et al., 1993). Assay results were quantitated by allowing the reaction to reach completion and then integrating the area under the kinetic curve relative to a blank reaction containing no antioxidant protection. The area under the curve was proportional to the concentration of all the antioxidants present in the sample (Esterbauer & Cheeseman, 1990).

Urine: A midstream urine sample (50 ml) from the first morning void was collected. Specific gravity was measured with a clinical refractometer (American Caduceus Industries, Inc.; Carle Place, NY). The remainder of urine was divided, frozen, and shipped to Genox Corporation (Baltimore, MD) and Data Chem Laboratories (Salt Lake City, UT) for the analyses of the following parameters:

1) Urine creatinine reacted with picric acid under alkaline conditions to form a Janovski complex (red). The absorbance of the color products measured at wavelengths from 480 to 520 nm was directly proportional to creatinine concentration in the sample.
2) Urine MDA+4-HNE reacted with Lipid Peroxidation Assay Kit (Calbiochem; San Diego, CA), Reagent 1 (R1) - N-methyl-2-phenylindole (10.3 mM) and Reagent 2 (R2) -

methanesulfonic acid (15.4 M) at 45°C. Condensation of one molecule of MDA or 4-HNE with two molecules of reagent R1 yields a stable chromophore with maximal absorbance at 586 nm (Esterbauer et al., 1991).

3) Urine MDA was mixed with 2,4-DNPH reagent. The samples were passed through pretreated C18 silica gel cartridges and eluted with acetonitrile. HPLC was performed to detect the urine MDA at 305 nm by Data Chem Laboratories, Salt Lake City, UT.
4) Urine 8-OHdG was determined using a Mab based ELISA monoclonal antibody kit (Genox; Baltimore, MD), a competitive in vitro enzyme linked immunosorbent assay for quantitative measurement of 8-OHdG (Shigenga & Ames, 1991). The optical density at a wavelength of 492 nm was read. By comparing the optical densities of the chromogenic signal of each sample with an internally run standard curve of known 8-OHdG concentrations, quantitation of 8-OHdG was achieved.
5) Urine 8-epi-$PGF_{2\alpha}$ was also measured using a Mab based ELISA monoclonal antibody kit (Genox; Baltimore, MD) (Morrow & Roberts, 1996). Optical density at a wavelength of 405 nm was read. Quantitation of 8-epi-$PGF_{2\alpha}$ was achieved by comparing the optical densities of the chromogenic signal of each sample to an internally run standard curve of known 8-epi-$PGF_{2\alpha}$ concentrations.

Statistical Analysis

Statistical analyses were performed using the SPSS statistical program (version 7.5; SPSS Inc.; Chicago, IL). Sample size was estimated by statistical power calculation setting the power = 0.80 and $p < 0.05$. Seventy-five subjects were enrolled in this study and divided into five groups of 15. This number of test subjects was determined by using the variance, S^2 of 4-HNE based on data collected for urine HNE for a prior study under similar conditions (Pfeiffer et al., 1997). However, due to injury, duty changes, and other uncontrollable reasons, we did not have the equal subjects in each group at the end of study.

Change-from-baseline comparisons between treatment groups were done using independent groups *t*-tests with Satterthwaite's degrees of freedom approximation (unequal variance *t*-test) (Snedecor & Cochran, 1980). This version of the *t*-test does not have the restrictive equal variances as assumption of the original Student's *t*-test.

The number of multiple comparisons was controlled somewhat by only comparing active treatment groups with the placebo group, thereby restricting the number of between group comparisons to four at any given follow-up time.

The Pearson correlation coefficient was used to test the relationships among the indices that were used in this study. All p values were for two-tailed tests ($p < 0.05$). All values shown represent the mean ± SD.

Results

Meteorological data

The meteorological data in Table 1 lists the daily low and high temperatures, pressure, and wind speed. The temperature ranged from 11°F (-11.7°C) to 38°F (3.33°C), the pressure ranged from 736 hPa (552 mm Hg) to 750 hPa (563 mm Hg), and the wind speed ranged from 1 mph to 24 mph during the 14-day field training. All field training took place upon a snow-base-

depth ranging from 4 ft to 8 ft. There was a wide temperature range in the training field. After sunset, the temperature was usually below the freezing level.

Descriptive subject characteristics and urine specific gravity

There were no statistically significant differences among treatment groups for age, body weight, or percent body fat at Day 0 versus Day 14 (Table 2). During the 2-week training period, subjects lost an average of 7.5 lb and lost an average of 1.6% body fat. Assuming euhydration, this suggests that weight loss was 40.8 percent from the fat free mass (FFM) and 59.2 percent from the fat mass (FM). Urine specific gravity was used to determine whether the subjects were well hydrated or dehydrated. If the urine specific gravity was higher than 1.030, the subject was considered to be dehydrated. Table 3 indicates that all subjects were well hydrated with an overall average for urine specific gravity of 1.020.

Daily energy expenditure and dietary intakes

Daily energy expenditure, daily energy intake, percentage of energy derived from macronutrients (fat, carbohydrate [CHO], and protein), daily dietary intake of vitamin E, A, C, iron, and zinc are shown in Table 4. There were no statistically significant differences among treatment groups in each case. Mean daily energy expenditure during 2 weeks of field training was approximately 5,500 kcal (23,000 kJ) estimated by intake-balance method.

Breath pentane assessment of oxidative stress

The change in breath pentane excretion from Day 0 to Day 14 was significantly different ($p < 0.05$) between the placebo and antioxidant mixture groups (Table 5) indicating that during the field training, the antioxidant mixture group had reduced breath pentane excretion compared with the placebo group. No other statistically significant differences were observed among treatment groups throughout the field training; therefore the remainder of the results section will only focus on the placebo and antioxidant mixture groups.

Both placebo and antioxidant mixture groups showed increasing oxidative stress after 14 days of field training (Table 5). The antioxidant mixture group had reduced breath pentane excretion compared with the placebo group ($p < 0.05$). Breath pentane increased 1600% in the antioxidant mixture group and 2314% in the placebo group (Table 5) between Day 0 and Day 14.

Table 1. Meteorological data: April 8 - 22, 1997

Day	Temp. Low (°F)[1,3]	Temp. High(°F)[2,3]	Pressure (hPa)[4,5]	Wind Speed (MPH)[4,5]
Tue, 4/4	23	24	741.1	19.3
Wed, 4,9	11	12	736.4	13.6
Thu, 4/10	11	15	740.1	6.3
Fri, 4/11	11	12	740.5	5.6
Sat, 4/12	11	16	742.7	1.0
Sun, 4/13	13	26	743.7	5.1
Mon, 4/14	20	26	743.0	6.2
Tue, 4/15	26	29	747.2	4.4
Wed, 4/16	34	35	748.1	12.4
Thu, 4/17	34	34	747.7	17.7
Fri, 4/18	33	33	744.4	19.1
Sat, 4/19	31	32	742.1	18.5
Sun, 4/20	31	38	749.0	16.2
Mon, 4/21	36	37	749.6	23.3
Tue, 4/22	33	35	746.3	11.4

[1] The lowest temperature recorded in the 24-hr day.
[2] The values are from the 6-7 AM time period.
[3] The data were recorded at Mammoth Lakes, California by Mammoth Mountain Ski Patrol, at 9,050 ft above sea level. Mammoth Lakes is approximately 46 miles due south of the MCMWTC training area, Silver Meadows, California (elevation: 9,200 ft).
[4] The data was recorded by United States Navy personnel on site at Silver Meadows, CA
[5] The values are the average from 8,000 to 9,000 ft.

Table 2. Subject characteristics before and after field training[1]

Treatment Group	n[2]	Age, year	Body Wt (lb) Day 0	Body Wt (lb) Day 14	Body Fat (%) Day 0	Body Fat (%) Day 14
Vitamin E	13	24.2±4.5	173.2±13.4	167.6±12.1	13.3±4.9	12.0±4.5
Vitamin A	15	24.4±7.5	174.9±19.6	166.1±18.8	13.4±5.7	12.3±5.0
Vitamin C	12	25.5±8.3	184.1±12.58	175.9±10.3	16.9±2.9	14.3±2.2
Antioxidant Mixture	13	25.8±7.4	175.9±18.7	168.6±16.3	14.3±5.4	11.9±3.9
Placebo	16	22.5±3.9	176.9±24.3	169.3±22.9	13.9±5.5	12.3±4.5
Average		24.4±6.4	176.9±18.4	169.3±17.0	14.3±5.1	12.5±4.1

[1] Values are means ± SD.
[2] n = number of subjects.

Table 3. Urine specific gravity before, during, and after the 14-day field training period [1]

Treatment Group	Day 0	Day 6	Day 12	Day 14
Vitamin E	1.017±0.01	1.023±0.01	1.026±0.01	1.024±0.01
Vitamin A	1.019±0.01	1.021±0.01	1.027±0.00	1.023±0.01
Vitamin C	1.016±0.01	1.021±0.00	1.022±0.01	1.020±0.01
Antioxidant Mixture	1.016±0.01	1.017±0.01	1.021±0.01	1.015±0.01
Placebo	1.018±0.01	1.024±0.01	1.027±0.01	1.025±0.01
Mean (all groups)	1.018±0.01	1.022±0.01	1.024±0.01	1.022±0.01

[1] Values are means ± SD.

Table 4. Daily energy expenditure and dietary intakes during field training[1]

	Treatment Group				
Nutrient	Vitamin E	Vitamin A	Vitamin C	Antioxidant Mixture	Placebo
Daily Energy Expenditure, kcal[2]	5267±1444	5670±835	5572±1586	5950±1516	5126±1115
Daily Energy Intake, kcal	3780±1113	3602±1004	3372±1193	4248±1121	3242±1380
Fat, % kcal	35.4±2.7	35.6±3.5	32.8±3.7	34.2±2.7	32.8±2.9
CHO, % kcal	49.3±3.8	49.8±6.0	50.7±10.5	50.4±6.3	50.5±8.2
Protein, % kcal	15.3±4.6	14.5±8.4	16.3±11.0	15.2±5.7	16.7±9.2
Vitamin E[3], mg	40.5±29.7	39.6±25.6	25.8±17.4	27.2±11.7	31.8±18.2
Vitamin A[4], RE	2206±1792	1743±916	1498±849	1711±772	1125±551
Vitamin C, mg	250±154	193±174	211±88	232±132	224±128
Iron, mg	23.2±4.6	21.8±6.5	25.2±12.8	24.5±7.5	19.9±8.8
Zinc, mg	12.0±3.6	12.0±5.8	12.9±5.8	13.9±7.3	10.2±2.9

[1] Values are means ± SD; these data do not take into account nutrients contributed by the antioxidant supplement.
[2] Energy expenditure was estimated by intake-balance method.
[3] 1.5 IU α-tocopherol = 1.0 mg Vitamin E.
[4] 1 RE = 6 mg β–carotene.
No significant differences were detected, $p > 0.05$.

Table 5. Breath pentane assessment of oxidative stress before and after the 14-day field training period[1]

Treatment Group	n[2]	Day 0	Day 6	Day 14	Comparison Day 0 to 14 (Paired t-test) p values	Change scores comparison Day 0 to Day 14 Tx vs. Placebo (Independent t-test); p values
Vitamin E	9	0.04 ± 0.06	0.49 ± 0.81	0.95 ± 1.20	0.033	0.23
Vitamin A	11	0.13 ± 0.09	0.62 ± 0.68	0.94 ± 1.10	0.024	0.15
Vitamin C	11	0.12 ± 0.15	0.47 ± 0.62	1.41 ± 1.97	0.534	0.67
Antioxidant Mixture	8	0.03 ± 0.06	0.29 ± 0.30	0.51 ± 0.64	0.071	0.03
Placebo	13	0.07 ± 0.08	0.29 ± 0.26	1.69 ± 1.61	0.003	

[1]Values are means ± SD; nmol/L
[2]n = number of subjects

Blood assessment of oxidative stress

Figures 1, 2, and 3 shows that after 14 days of training, there were no statistically significant differences found between the placebo and antioxidant groups. Oxidative stress tended to increase (serum MDA + 4-HNE and plasma LPO) and antioxidant capacity (ORAC) decreased. Serum MDA + 4-HNE increased 7% in the antioxidant mixture group and 4% in the placebo group (Figure 1) between Day 0 and Day 14 (ns). Plasma LPO increased 61% in the antioxidant mixture group and 83% in the placebo group (Figure 2) between Day 0 and Day 14 (ns). Serum ORAC decreased 4% in the antioxidant mixture group and 1% in the placebo group (Figure 3) between Day 0 and Day 14 (ns).

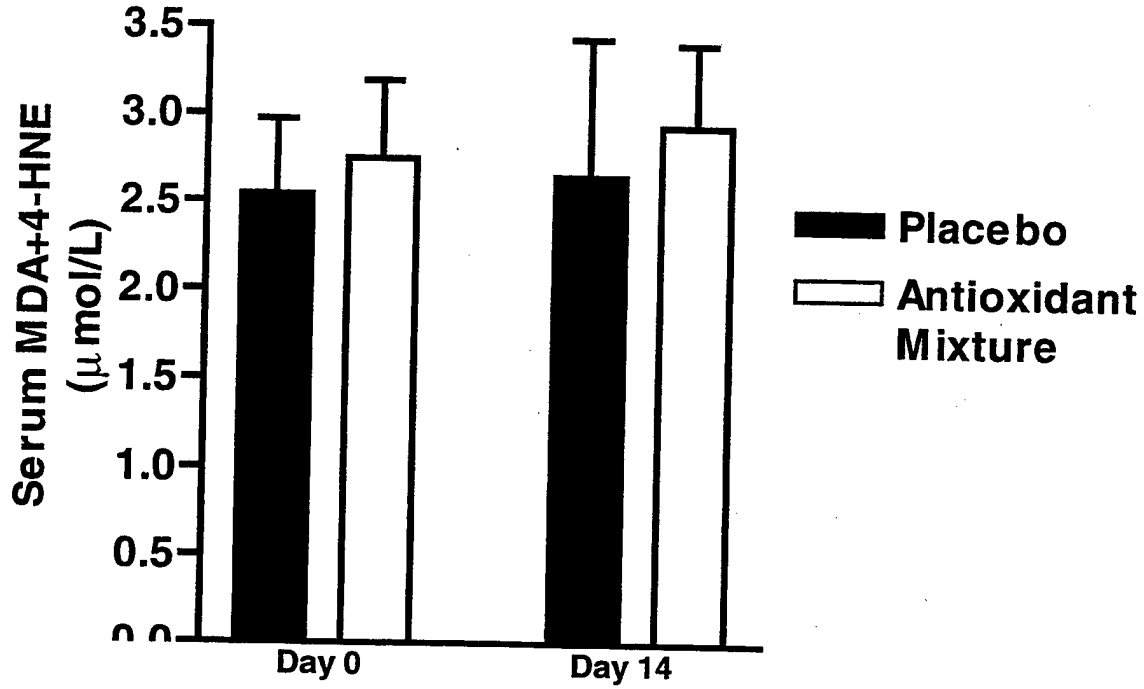

Figure 1. Serum MDA+4-HNE before and after the 14-day field training period (antioxidant mixture group vs. placebo group). No significant differences were detected, $p > 0.05$.

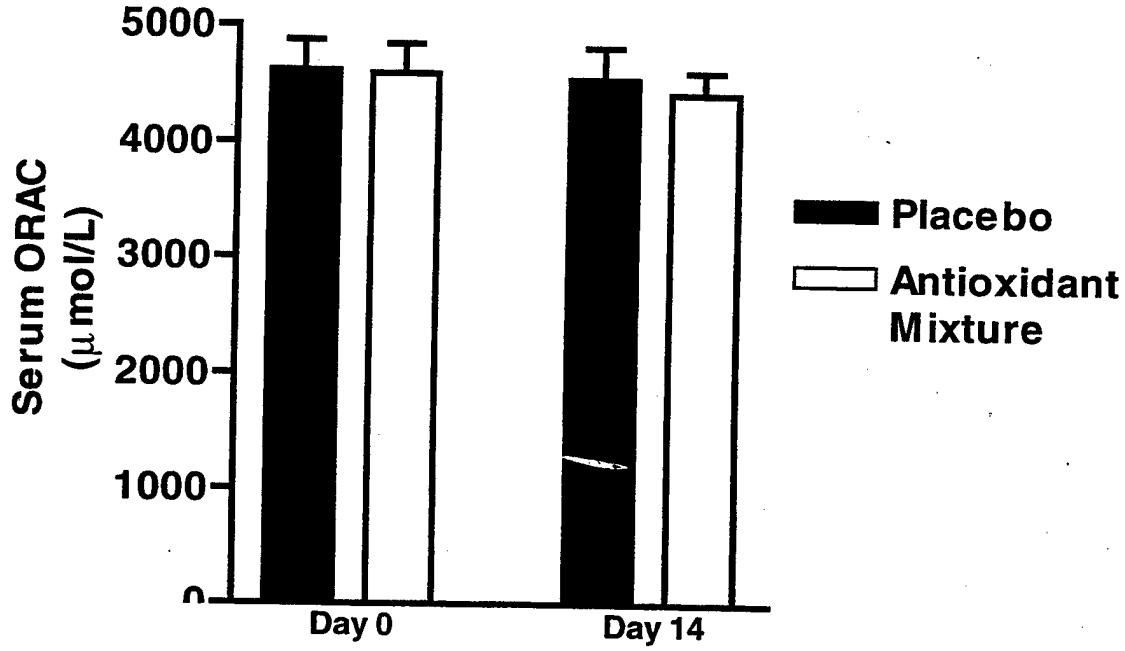

Figure 2. Serum ORAC before and after the 14-day field training period (antioxidant mixture group vs. placebo group). No significant differences were detected, $p > 0.05$.

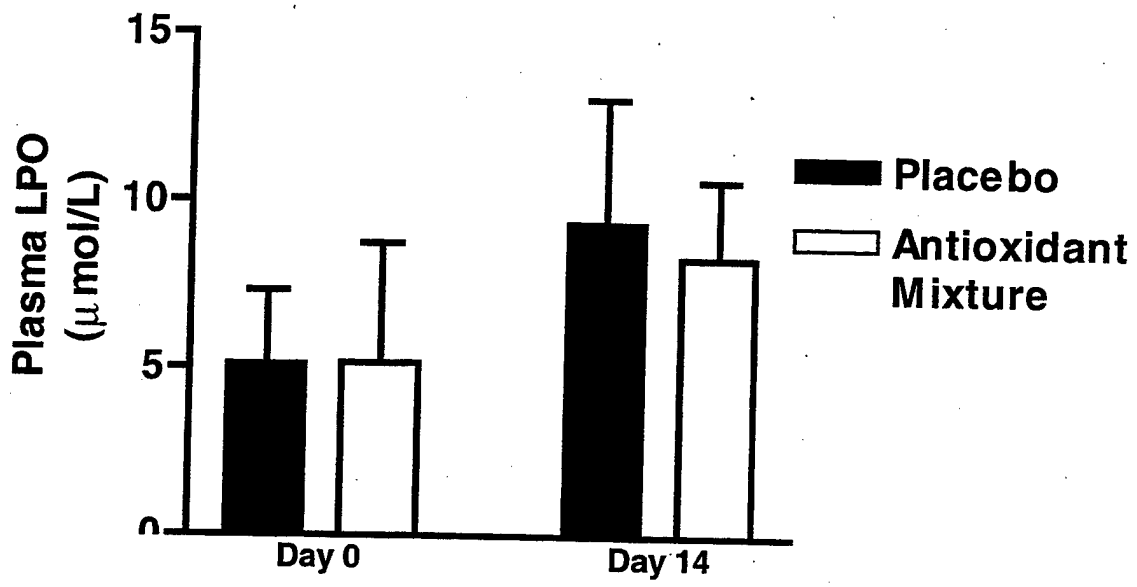

Figure 3. Plasma LPO before and after the 14-day field training period (antioxidant mixture group vs. placebo group). No significant differences were detected, $p > 0.05$.

Serum antioxidant vitamin profile

Tables 6 and 7 show that after supplementation, in either the individual vitamin or the combination vitamins and minerals mixture groups, the subject's blood reflected the expected elevated serum vitamin profile according to the supplements they were consuming. This also indicated that the subjects actually took the supplements that they were to consume.

Table 6. Serum carotenoids profile in Camp Pendleton (sea level) and at the end of field training (6,700 ft).

Treatment Group		α-Carotene (μg/ml)	β-Carotene (μg/ml)	Retinol (μg/ml)	Retinyl Palmitate (μg/ml)
Vitamin E	Pendleton	0.01±0.010	0.12±0.045	0.64±0.132	0.03±0.032
(n = 12)	Day 14	0.03±0.021	0.12±0.053	0.54±0.119	0.03±0.030
Vitamin A	Pendleton	0.02±0.009	0.12±0.039	0.61±0.130	0.02±0.024
(n = 14)	Day 14	0.05±0.046	0.31±0.262	0.50±0.145	0.02±0.024
Vitamin C	Pendleton	0.02±0.011	0.12±0.027	0.59±0.114	0.03±0.035
(n = 12)	Day 14	0.02±0.014	0.12±0.034	0.45±0.079	0.03±0.015
Antioxidant Mixture	Pendleton	0.03±0.034	0.22±0.240	0.53±0.132	0.03±0.041
(n = 13)	Day 14	0.04±0.017	0.27±0.176	0.47±0.100	0.03±0.020
Placebo	Pendleton	0.01±0.010	0.11±0.035	0.56±0.124	0.02±0.021
(n = 15)	Day 14	0.02±0.019	0.11±0.035	0.45±0.113	0.02±0.019

Table 7. Serum vitamin E and vitamin C profile at Camp Pendleton (sea level) and at the end of field training (6,700 ft).

Treatment Group		α-Tocopherol (μg/ml)	γ-Tocopherol (μg/ml)	δ-Tocopherol (μg/ml)	Vitamin C (μg/ml)
Vitamin E	Pendleton	7.76±2.70	0.14±0.084	2.52±0.93	19.37±2.96
(n = 12)	Day 14	10.63±2.61	0.10±0.090	1.69±0.76	23.27±3.21
Vitamin A	Pendleton	7.44±1.65	0.14±0.120	2.44±0.97	19.60±3.80
(n = 14)	Day 14	7.40±1.26	0.07±0.065	3.93±6.40	22.08±4.17
Vitamin C	Pendleton	7.85±2.18	0.15±0.073	2.32±0.76	21.80±3.54
(n = 12)	Day 14	7.63±2.16	0.08±0.075	1.95±0.55	23.95±2.83
Antioxidant Mixture	Pendleton	7.58±3.44	0.07±0.072	1.70±0.59	21.69±5.19
(n = 13)	Day 14	12.42±4.16	0.03±0.048	1.12±0.51	24.38±4.78
Placebo	Pendleton	7.32±1.73	0.10±0.068	1.77±0.62	20.59±3.63
(n = 15)	Day 14	8.12±1.80	0.10±0.068	2.10±0.60	21.72±4.80

Urine assessment of oxidative stress

Excretion patterns of aldehydes and 8-OHdG in the urine (Figures 4, 5, and 6) all indicated a general increase in levels of these indicators over time, but there were no significant differences between the placebo and antioxidant groups. Urine MDA increased 203% in the antioxidant mixture group and 229% in the placebo group (Figure 4) between Day 0 and Day 14 (ns). Urine total aldehydes decreased 6% in the antioxidant mixture group and increased 50% in the placebo group (Figure 5) between Day 0 and Day 14 (ns). Urine 8-OHdG increased 14% in the antioxidant mixture group and 50% in the placebo group (Figure 6) between Day 0 and Day 14 (ns). However, the 8-epi-PGF_{2a} responded somewhat differently (Figure 7). Urine 8-epi-PGF_{2a} inexplicably decreased 55% in the antioxidant mixture group and 54% in the placebo group (Figure 8) between Day 0 and Day 14 (ns).

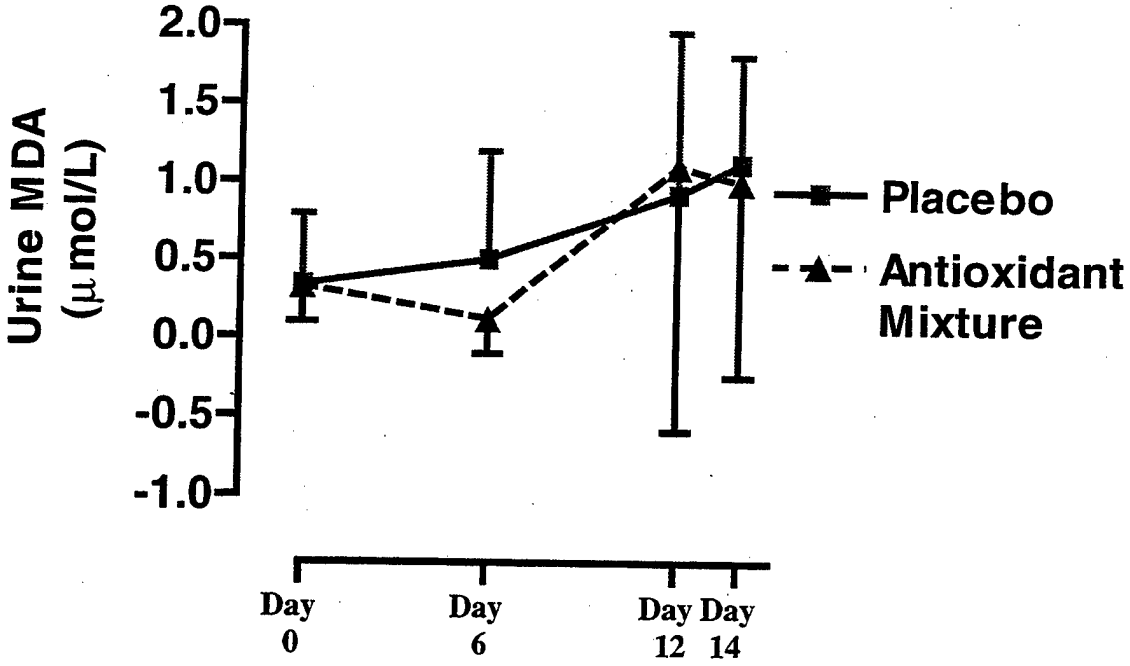

Figure 4. Urine MDA before, during, and after the 14-day field training period (antioxidant mixture group vs. placebo group).

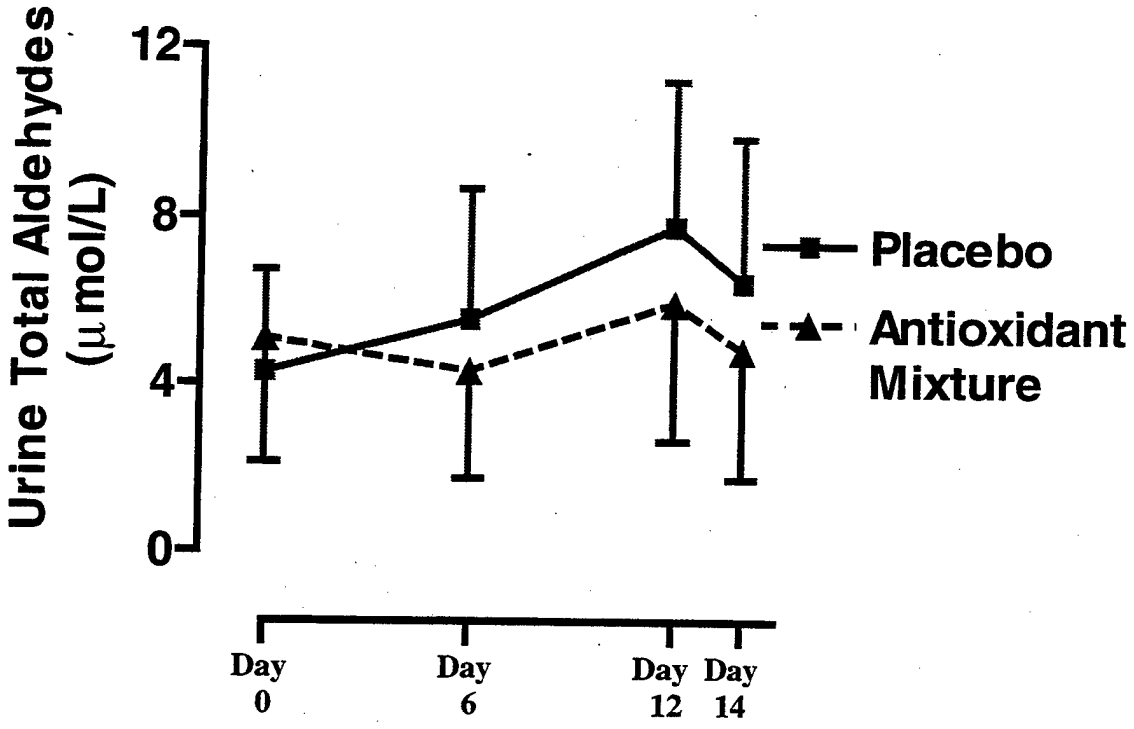

Figure 5. Urine total aldehydes before, during, and after the 14-day field training period (antioxidant mixture group vs. placebo group).

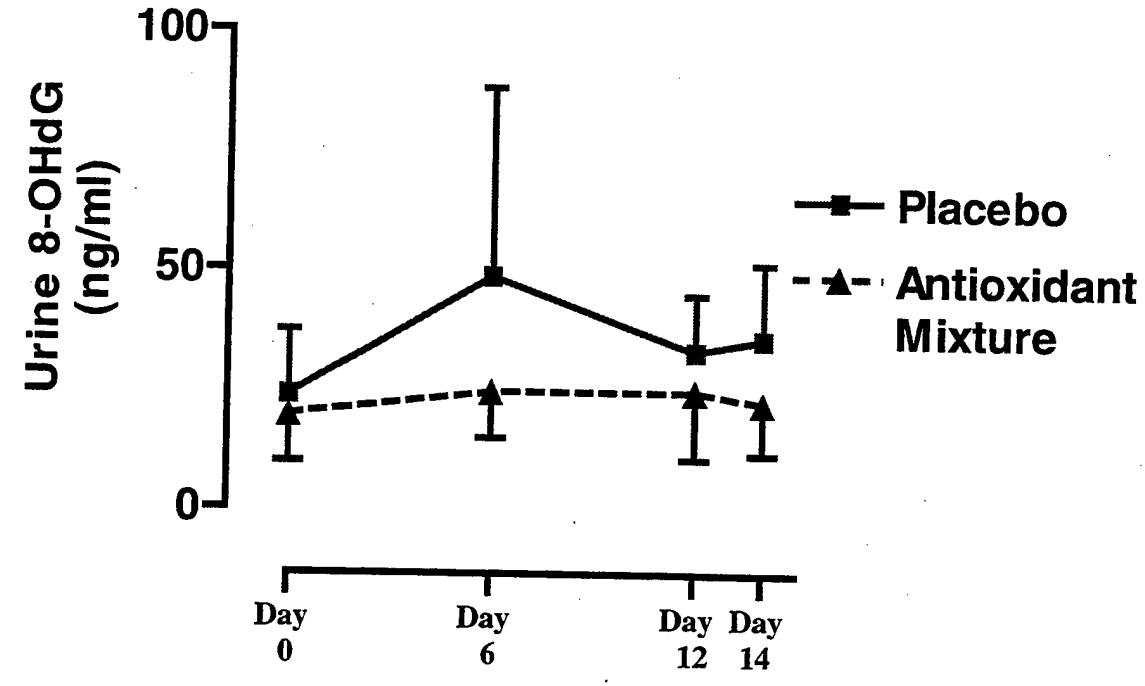

Figure 6. Urine 8-OHdG before, during, and after the 14-day field training period (antioxidant mixture group vs. placebo group).

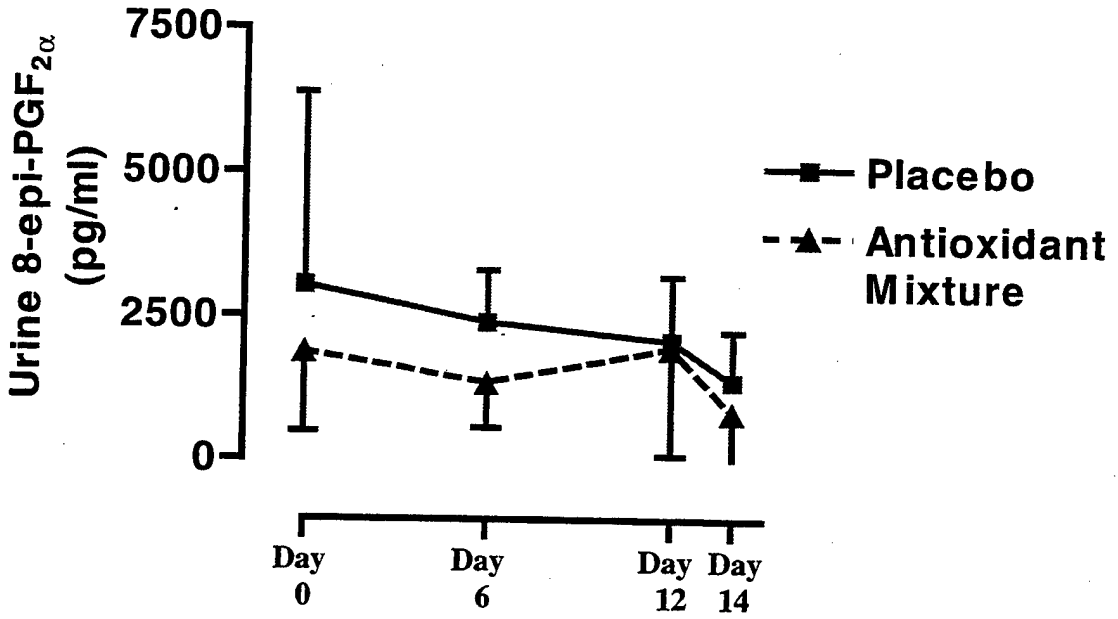

Figure 7. Urine 8-epi-$PGF_{2\alpha}$ before, during, and after the 14-day field training period (antioxidant mixture group vs. placebo group).

Correlation tests among the indices of oxidative stress

Table 8 shows that there were statistically significant relationships among some of the indices of oxidative stress at Day 14.

Table 8. Pearson correlation test among the indices of oxidative stress at Day 14.

	2	3	4	5	6	7	8
1.Breath Pentane	-.253* (n = 66)	.088 (n = 66)	-.019 (n = 66)	-.196 (n = 66)	.062 (n = 66)	-.098 (n = 66)	-.234 (n = 65)
2. Plasma LPO		-.008 (n = 70)	.078 (n = 70)	-.049 (n = 70	-.331** (n = 70)	-.092 (n = 66)	.227 (n = 69)
3. Serum MDA			-.002 (n = 70)	-.082 (n = 70)	-.069 (n = 70)	.011 (n = 66)	.095 (n = 69)
4. Serum ORAC				-.146 (n = 70)	-.265* (n = 70)	-.335** (n = 66)	.065 (n = 69)
5. Urine Total Aldehydes					.086 (n = 70)	.552** (n = 66)	.218 (n = 69)
6. Urine MDA						.259* (n = 66)	.120 (n = 69)
7. Urine 8-OHdG							-.335** (n = 65)
8. Urine 8-epi-$PGF_{2\alpha}$							

* $p < 0.05$. ** $p < 0.01$.

Discussion

Evidence of oxidative stress was exhibited in all treatment groups after 2 weeks of training as evidenced by higher breath pentane ($p < 0.05$), urine MDA ($p < 0.05$), urine total aldehydes, urine 8-OHdG ($p < 0.05$), and blood LPO ($p < 0.05$). The only exception was the oxidized urine 8-epi-$PGF_{2\alpha}$, which did not increase. The individual antioxidant nutrient treatments were relatively ineffective in reducing these indicators of oxidative stress.

The results of this study agree with a similar study conducted by Simon-Schnass and Pabst (1988), in which the authors reported that after 2 weeks of field training at moderate altitude, breath pentane was significantly increased. In this study, the placebo group showed a significant increase in breath pentane, while in the antioxidant mixture group, breath pentane was

significantly lower ($p < 0.05$) than the placebo group. However, this protective effect was not seen in the individual vitamin supplementation groups.

The results of the present study can be compared with a previous study conducted by Pfeiffer et al. (1997) in which there were only two groups, a placebo and an antioxidant mixture group, but utilizing the same Marine field-training model. Plasma LPO and urine 8-OHdG indices used in this study, were measured by Pfeiffer et al. (1997) and indicated no significant antioxidant mixture (treatment) effects after 14-day field training.

A possible explanation why a greater antioxidant effect was not seen in this study and Pfeiffer's study was that the military diet is already relatively high in dietary antioxidants (approximately 150% higher in vitamin E, 400% higher in vitamin A, and 700% higher in vitamin C than the RDA); hence, supplemental antioxidants might not be expected to show a great effect. Another reason may be that the military subjects were highly physically trained. The subjects may already have developed a highly efficient antioxidant enzyme defense and, along with their diet, may have achieved significant protection against oxidative stress. This explanation agrees with the serum ORAC data, measuring total serum antioxidant activity, which did not indicate a lack of serum antioxidants.

Also, breath pentane may reflect immediate or short-term oxidative stress. Other indices may reflect accumulated or longer-term oxidative stress. Hence, the timing of the collection of breath pentane may be important in its interpretation and utility in determining the relationship to overall oxidative stress. To see the same results from the other indices, a 2-week duration may not be long enough to reflect the results seen with breath pentane.

The reason urine 8-epi-$PGF_{2\alpha}$ did not respond as expected might be explained by the way the urine sample was collected. The first-void morning urine sample may not represent the actual 24-hr excretion of urine 8-epi-$PGF_{2\alpha}$. Twelve hours of urine collection might be a better alternative to be used in related field studies. Alternatively, 8-epi-$PGF_{2\alpha}$ may not be a true indicator of oxidative stress under the conditions of this study.

Overall, it may be incorrect to conclude that the vitamin and mineral mixture failed to perform better as an antioxidant than the placebo when compared with the rest of the oxidative stress indices (except the breath pentane). The variability in the data collected was much larger than the variance estimate used in the sample size calculation. Since the resulting statistical power was much smaller than 0.80, it was less likely that statistical significance would be achieved, even if true in the population. The antioxidant mixture group, compared with the placebo group, displayed a smaller breath pentane change from the Day 0 (baseline) to Day 14 ($p = 0.032$). This observed effect had the greatest post hoc power of all the observed effects in the study, but the power was only 0.52. This indicates that the variability of data collected in field studies can be quite large, probably the result of unavoidable individual influences such as amount of exercise, degree of prior physical training, sunlight exposure, prior dietary habits, individual variation in selection of food items, smoking, etc.

Conclusions

Breath pentane, blood LPO, urine MDA, urine total aldehydes, and urine 8-OHdG generally reflected increased oxidative stress; however, urine 8-epi-$PGF_{2\alpha}$ for some unknown reason did not. Breath pentane especially seemed to reflect an increased level of oxidative stress and its attenuation by an antioxidant mixture by Day 14. Compared with the antioxidant mixture, individual antioxidant treatments did not have as great an effect on reducing indicators of oxidative stress. Not all the indices in breath, blood, and urine supported the same conclusion for the first hypothesis. Actually, for some indicators, individual vitamin antioxidant treatment groups also exhibited significant increases of oxidative stress. Only the breath indicator, pentane, appeared to show the treatment effect of the antioxidant mixture, supporting the second research hypothesis for this study. In conclusion, people who work and recreate at moderate altitude may be at an increased risk for oxidative stress damage due to free radical production. However, the results of this study do not definitively establish this as a cause-and-effect relationship. A corresponding relationship between biochemical indicators of oxidative stress in breath, blood, and urine could not always be ascertained, indicating the importance of including indicators of oxidative stress from more than one source. The lack of agreement between indicators suggests several possibilities: (1) either some indicators are not directly related to oxidative stress, (2) the level of oxidative stress generated in this study was not sufficient to trigger their increase, (3) the timing of sampling was not appropriate for each index, or (4) the duration of supplementation was insufficient to moderate all indices.

Implications

Many people work and/or recreate at moderate altitudes throughout the world. Are these people at an increased risk for oxidative damage from free radical production? Would they benefit from an antioxidant supplement while they are working at such an altitude? What is the best method to measure oxidative stress in humans to determine if antioxidant supplementation is needed or beneficial? These questions remain to be answered in research on antioxidant supplementation for recreational people or athletes. This research is applicable to any population engaged in camping, backpacking, or cross-country activities in the mountains. This study indicates that oxidative stress does increase during work at moderate altitudes and suggests that an antioxidant supplement may be beneficial. However, the relationships among various indicators of oxidative stress are not clear; hence, an exact dietary recommendation could not be made. A prophylactic antioxidant supplement mixture may be good "dietary insurance" for people working or recreating at moderate altitude. Therefore, it is important that the relationship between these indicators and measures of oxidative stress be explored further. This knowledge would permit conclusions to be drawn as to the need for supplemental antioxidants in the diet of recreational/working people.

Limitation and Future Research

The results of this study, while generally applicable to a model of hard physical work in an outdoor environment, is specific only to U.S. Marines who participated in the cold weather moderate altitude field training at the MCMWTC. Although each subject acted as their own control, there are several factors that influence the individual subject's oxidative stress

parameters, including physical activity level, diet habit, tobacco use, sunscreen use, and environment factors. We did not collect 24-hr urine samples in this study due to the difficulty of obtaining quantitative collections in the field and large number of test subjects involved. Due to varying duties, it is unknown if the groups shared similar physical load during the entire 14-day training period. Finally, under no supervision, it is dependent upon the subjects to honestly take their daily supplements, although the measured vitamin levels suggested that they did.

Despite the limitations indicated above, the overall advantages of the model used in this study are far better than disadvantages. In future studies, 12-hr urine collection instead of the first void sample may be a reasonable alternative. Also, repeated measures of breath pentane at shorter intervals may be a more reliable method to determine the actual oxidative stress of the test subjects. If the future researchers may want to study a different model in which the subjects do not have diets already fortified as in these military subjects. Finally, more than one source of the markers of oxidative stress should be included in the future related studies to assess the oxidative stress.

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REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE & DATE OVERED
	2-24-99	FINAL, JUL 98–MAY999

4. TITLE AND SUBTITLE
The effect of vitamin supplementation on oxidative stress during strenuous cold weather training at moderate altitude.

5. FUNDING NUMBERS
Program Element: 63706N
Work Unit Number: M0096

002.6603

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8. PERFORMING ORGANIZATION
Report 99-6

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Code: BUMED-26
2300 E Street NW
Washington, DC 20372-5300

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE
A

13. ABSTRACT (Maximum 200 words)

This study quantified the effect of α-tocopherol, ascorbic acid, and β-carotene, singly and in combination, on oxidative stress (OS) during training at moderate altitude. OS was quantified by indirect measures of lipid peroxidation and DNA alteration. The efficacy of the vitamins was assessed as reduced urinary, blood, and breath indices of lipid peroxidation. Seventy-five Marines were assigned to 1 of 5 groups for a double-blind, 14-day study. The groups took, daily: (1) 500 mg ascorbic acid; (2) 400 IU α-tocopherol; (3) 20,000 IU β-carotene; (4) 500 mg ascorbic acid, 400 IU α-tocopherol, 20,000 IU β-carotene, 100 μg selenium, and 30 mg zinc; or (5) 1,000 mg oyster shell calcium (placebo). OS indicators included plasma lipid peroxides and oxygen radical absorption capacity, urine malondialdehyde and 8-hydroxy-deoxy-guanosine, and breath pentane. Samples were collected at sea level, Day 0 at 6,700 ft, Days 6 and 12 at 9,200 ft, and Day 14 at 6,700 ft. All measures generally reflected increased OS. All groups showed increased OS, indicated by breath pentane; however, the mixture resulted in a significantly smaller increase. Individual antioxidants were relatively ineffective in reducing the indicators of OS. Other measures indicated OS was increased but did not differentiate as did breath pentane.

14. SUBJECT TERMS
Oxidative stress, moderate altitude, vitamin supplementation, cold weather training.

15. NUMBER OF PAGES
29

16. PRICE CODE

17. SECURITY CLASSIFI-CATION OF REPORT	18. SECURITY CLASSIFI-CATION OF THIS PAGE	19. SECURITY CLASSIFI-CATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	**Unclassified**	**Unclassified**	**Unclassified**